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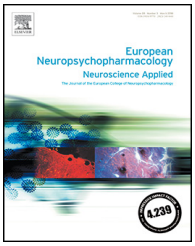

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Chronic dietary changes in n-6/n-3 polyunsaturated fatty acid ratios cause developmental delay and reduce social interest in mice

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Abstract
Polyunsaturated fatty acids (PUFAs) are one of the main cellular building blocks, and dietary changes in PUFA composition are proposed as a potential route to influence brain development. For example, initial studies indicated that there is a relation between blood omega-6(n-6)/omega-3(n-3) PUFA ratios and neurodevelopmental disease diagnosis. To study the consequences of dietary n-6/n-3 PUFA ratio changes, we investigated the impact of a n-3 supplemented and n-3 deficient diet in developing BTBR T + Itpr3tf/J (BTBR) - a mouse inbred strain displaying Autism Spectrum Disorder (ASD)-like symptomatology - and control C57BL/6J mice.

Abbreviations: ADHD, Attention Deficit Hyperactivity Disorder; ASD, Autism Spectrum Disorders; BL6, C57BL/6J mouse; BPS, Balano-Preputial Separation; BTBR, BTBR T+Itpr3tf/J; DHA, Docosahexaenoic acid, 22:6n-3; EFA, Essential Fatty Acids; EPA, Eicopentaenoic acid, 20:4n-3; EPM, Elevated Plus Maze; eSH, Extended SHIRPA screen; GLA, Gamma-linolenic acid, 18:3n-6; HC, Home cage screen; LC/MS, Liquid chromatography – mass spectrometry; n-3, Omega-3; n-6, Omega-6; OF, Open Field; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; PUFA, Polyunsaturated fatty acids, RR, Rota-Rod.

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This study showed that pre- and postnatal changed dietary n-6/n-3 ratio intake has a major impact on blood and brain PUFA composition, and led to delayed physical development and puberty onset in both strains. The PUFA induced developmental delay did not impact adult cognitive performance, but resulted in reduced social interest, a main ASD behavioral feature. Thus, both chronic dietary n-3 PUFA supplementation and depletion may not be beneficial.

1. Introduction

Polyunsaturated fatty acids (PUFAs) are main components of phospholipids and part of each cell (Rustan and Drevon, 2005). These building blocks are important for cell growth and development and influence multiple processes in the body. PUFAs can be categorized in 2 different classes, namely, omega-6 (n-6) and omega-3 (n-3) PUFAs, depending on the start of the first double carbon bond (Jenski and Stillwell, 2001; Rustan and Drevon, 2005). N-6 and n-3 PUFA precursors compete for the same enzymes in the PUFA pathway and, as a consequence, high intake of one, results in lower levels in the pathway of the other. The amount of all forms of n-6 and n-3 PUFAs in the body is mainly dependent on dietary precursor and mainly longer chain PUFA intake, as the body cannot synthesize the n-6 and n-3 essential fatty acids (EFAs) itself (Schmitz and Ecker, 2008). The overall effects of these PUFAs appear not dependent on individual levels but rather on the ratio of omega-6/omega-3 (n-6/n-3) PUFAs (Simopoulos, 2011).

Prenatally, the unborn child accumulates PUFAs in the brain primarily during the last trimester of pregnancy (Bernardi et al., 2012; Hamosh and Salem Jr., 1998) and the level of accretion is dependent on maternal PUFA intake (Greenberg et al., 2008; Jensen, 2006). This period is assumed to be most critical for cognitive development, as n-3 PUFA docosahexaenoic acid (n-3 DHA; 22:6n-3) accretion in the last trimester is highest due to increased neurogenesis and cell maturation (Bernardi et al., 2012; Greenberg et al., 2008; Jensen, 2006; Riediger et al., 2009). Postnatally, the highest accretion of n-3 DHA is in the first six months of life; about 50% of the total n-3 DHA body accumulation during this period takes place in the brain (Guesnet and Alessandri, 2011). This indicates the importance of n-3 DHA presence during brain development (Bernardi et al., 2012; Guesnet and Alessandri, 2011) and changing these lipid concentrations may lead to problems with lipid profiles and signaling (Wong and Crawford, 2014). The impact of these alterations in PUFA concentration and ratio is dependent on timing and duration of this PUFA change (Jensen, 2006). Thus, n-3 PUFAs are nutrients needed for optimal nervous system development and changing these might be detrimental for brain development (Guesnet and Alessandri, 2011; Wong and Crawford, 2014).

Historically, our dietary composition changed significantly, especially since the introduction of artificially produced n-6 rich vegetable oils and the reduction in dietary cholesterol intake. These vegetable oils were cholesterol-free resulting in a strong increase of dietary n-6 PUFA, but stable n-3 PUFA intake (Gerrior et al., 2004; Hiza and Bente, 2011). Recent studies have shown that lower blood levels of n-3 PUFAs have been found in patients with depression, dyslexia, schizophrenia, attention-deficit hyperactivity disorder (ADHD) or autism spectrum disorders (ASD) compared to controls (Gow and Hibbeln, 2014; Perica and Delas, 2011; Richardson and Ross, 2000), suggesting that a higher n-6/n-3 PUFA ratio and thus proportionally reduced n-3 PUFA levels in the body is related to the prevalence of brain disorders (Haag, 2003; Riediger et al., 2009). For ASD, a neurodevelopmental disorder characterized by developmental delay and deficits in social interaction and stereotyped behaviors (American Psychiatric Association, 2013), we and others recently hypothesized that the increasing rise in autism prevalence (Blaxill, 2004; Centers for Disease Control and Prevention, 2014, 2012) parallels the disturbed dietary n-6/n-3 PUFA ratio following the introduction of these artificial oils (Neggers, 2014; van Elst et al., 2014). Related to this hypothesis, there is evidence that n-3 DHA is a blood serum biomarker for ASD; lower levels of n-3 DHA could predict ASD diagnosis (Bell et al., 2000; Brown et al., 2014; Wang et al., 2016). However, despite a variety of studies on the influence of PUFAs on development (Riediger et al., 2009), it is still uncertain whether changes in PUFA ratio, especially with a n-3 supplemented or n-3 deficient feeding, is beneficial for brain functioning (Bazinet and Chu, 2014; Dyall and Michael-Titus, 2008; Simopoulos, 2006). Furthermore, the question remains whether adding n-3 PUFAs to a diet should be used as an alternative treatment for this neurodevelopmental disorders, such as ASD (Brondino et al., 2015; Hanson et al., 2007; Lofthouse et al., 2012; Ranjan and Nasser, 2015).

Animal studies can provide insights into the contribution of n-6/n-3 PUFA ratio on brain and behavioral development using controlled interventions (see Supplementary Table 4). Here we study the contributions of two different dietary n-6/n-3 PUFA ratios, namely by increasing and by decreasing this dietary ratio across all developmental stages, to determine how these PUFA ratios can influence development, cognitive functioning and behavioral expression. For these studies, we investigated the developmental impact of PUFA dietary composition in the BTBR mouse inbred strain, a commonly used ASD mouse model displaying the phenotypic features of disturbed social interaction and restrictive and repetitive behavior (McFarlane et al., 2008; Meyza and Blanchard, 2017; Molenhuis et al., 2014; Pearson et al., 2011). In parallel, identical dietary changes in n-6/n-3 PUFA ratios were studied in the C57BL/6J mouse inbred strain, a commonly chosen reference strain (e.g., Molenhuis et al., 2014; Moy et al., 2004).

2. Experimental procedures

2.1. Animals

C57BL/6J Mice were obtained from Charles River (Sulzfeld, Germany) and BTBR T^{+} *Itpr3*^{tf}/J (BTBR) mice from The Jackson Laboratory (Bar Harbor, USA). These inbred mice were used for breeding at the University Medical Center Utrecht, the Netherlands. Their male offspring was generated for the experiments. Male mice were weaned at postnatal day 21 (P21), ear punched for identification and socially housed with litter mates in groups of 2–5 mice per cage. All mice were bred and housed under a 24 h reversed light-dark cycle (white lights on from 19.00 to 7.00 h). All experiments were approved by the ethical committee for animal experimentation of the University Medical Center Utrecht and performed according to the University Medical Center institutional guidelines that are in full compliance with the European Council Directive (86/609/EEC).

2.2. Diets

Food and water was provided ad libitum. Both dams and offspring were fed a diet with different n-6/n-3 ratio in chow (AIN-93G based), depending on the experimental group that they were assigned to (e.g., either n-3 supplemented or n-3 deficient diets). Dams started the diet 1–2 weeks before pairing with a male and it was kept throughout offspring's life. Diets were custom made at Special Diets Services (SDS; Technilab-BMI bv, Someren, Netherlands). AIN-93G was used as the control diet (Control) in the experiment (Ratio 8.4:1). The n-3 deficient diet (n-3 Def) was manufactured by replacing all soya bean oil (7%) by sunflower oil (7%) (1.1% n-3 and 67.5% n-6; ratio 235:1). The n-3 supplemented diet (n-3 Supp) was manufactured by replacing the 7% soya bean oil partially by 5.8695% oil compound, containing 50% DHA (Docosahexaenoic acid), 7% EPA (Eicosapentaenoic acid) and 10% GLA (Gamma-linolenic acid), 19.6 g Vitamin E, ratio 1:1.3) (Vifor Pharma, Glattbrugg, Switzerland). All diets were analyzed after preparation for fatty acid composition (see Table 1).

2.3. Blood sampling

Blood samples were taken at 4 developmental time points (4, 6, 8 and 10 week old mice covering, respectively, preadolescence, adolescence, early adulthood and adulthood (Molenhuis et al., 2014). Samples were collected by making a small cut into the tail with a razorblade (GEM Scientific, Bradford, UK). For this procedure, mice were kept under an inverted grid to prevent stress from fixation. Blood samples were always taken at the same time of the day.

First, blood was collected on special Spot Saver Cards (PerkinElmer 226 Spot Saver Cards, Whatman, GE Healthcare, UK) treated with antioxidant (Butylated hydroxytoluene (BHT)) (Sigma, Dorset, UK) for fatty acid analyses. After a 3h drying period at room temperature, cards were stored in foil bags (Whatman, GE Healtcare, UK) with desiccant in the −20 °C freezer until analyses. Second, blood was collected on Blood Glucose Test strips to measure Glucose levels with a Glucose measure system (FreeStyle Precision Neo H, Abbot Diabetes Care, Oxon, UK).

2.4. Lipid extraction and fatty acid analysis on blood spots

Dried blood spots were automatically treated with a PAL HTX-*xt* robot, which prepares and purifies Fatty Acid Methyl Esters (FAME). FAME was then used for Gas-liquid chromatography (GLC) using a ThermoFisher Trace GC 2000 (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60 m × 0.32 × 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150 °C at 40 °C/min, then to 195 °C at 1.5 °C/min and finally to 220 °C at 2 °C/min. Individual methyl esters were identified by reference to published data (Ackman et al., 1980; Bell et al., 2011). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy).

2.5. Lipidomics

Brain dissection was performed on P21 BTBR. Mice were decapitated and brains were quickly removed and frozen on dry ice. The brain was stored in the −80 °C freezer until use. Lipids were extracted from 5% brain homogenates in PBS according to the method of Bligh and Dyer (Bligh and Dyer, 1959). Separation of polar classes was performed as described elsewhere (Jeucken and Brouwers, 2016) In brief, lipids were injected in 10 μL of chloroform/methanol (1:1, v/v) on a Kinetex HILIC column (Phenomenex, Torrance, CA). Elution was performed with a gradient from ACN/acetone (9:1, v/v) to ACN/H2O (7:3, v/v). Eluting phospholipids were detected by mass spectrometry using positive mode atmospheric pressure chemical ionization and intensities were used for analysis. For ether linked lipid species, the plasmalogen subclass was assumed.

2.6. Behavioral procedures

Before each behavioral task, animals were transferred to the test-room and habituated for at least one hour. All mice were tested from early adolescence until adulthood. The order of the experiments is similar to the order of experiments described below. During development, mice were tested once per time point (4, 6 and 8 weeks old) in the same set-up. From 10 weeks on mice were exposed to a behavioral test battery. The order of experiments was identical to the order mentioned below. No more than 2 experiments were performed in the same week. For social experiments, there was 1 week in between. After each trial in each experiment, the set-up was cleaned using Trigene solution (0.5%; Tristel Solutions Ltd, UK).

Table 1 The dietary composition of each individual diet. Results are averages of multiple batches of chow.

Basic dietary composition		Control	n-3 Supp	n-3 Def
Maize starch		39,75	39,75	39,75
Casein		20	20	20
Maltodextrin		13,2	13,2	13,2
Sucrose		10	10	10
Cellulose		5	5	5
Mineral mix		3.6	3.6	3.6
Vitamin mix		1	1	1
L-cystine		0,3	0,3	0,3
Choline Bitartrate		0,25	0,25	0,25
Antioxidant.			Vitamin E	
FAT		**7,9**	**8,3**	**8**
Caloric content (Kcal/g)		**3.895**	**3.896**	**3.893**
Result as % in the diet (actual fatty acids)	Name	Result as % in the diet	Result as % in the diet	Result as % in the diet
C18:1 (n6) cis	cis-12-Octadecanoic Acid	0,001	0,002	0,001
C18:1 (n6) trans	Trans-12-Octadecanoic Acid	0,001	0,003	0,001
C18:2 (n6) cis	Linoleic Acid	3,583	1,641	4,022
C18:2 (n6) trans	Trans Linolelaidic Acid	0,001	0,001	0,001
C18:3 (n3) cis	Alpha-Linolenic Acid (ALA)	0,418	0,102	0,014
C18:3 (n6) cis	Gamma-linoleic Acid (GLA)	0,001	0,108	0,001
C18:4 (n3) cis	Stearidonic Acid	0,001	0,018	0,001
C20:2 (n6) cis	Cis-11,14-Eicosadienoic Acid	0,003	0,020	0,001
C20:3 (n3) cis	Cis-11,14,17-Eicosatrienoic Acid	0,001	0,015	0,001
c20:3 (n6) cis	Cis-8,11,14-Eicosatrienoic Acid	0,001	0,008	0,001
C20:4 (n3) cis	Cis-8,11-14,17-Eicosatetraenoic Acid	0,001	0,027	0,001
C20:4 (n6) cis	Arachidonic Acid	0,008	0,108	0,002
C20:5 (n3) Cis	Eicosapentaenoic Acid (EPA)	0,008	0,354	0,007
C22:2 (n6) cis	Docosadienoic Acid	0,001	0,303	0,001
C22:4 (n6) cis	Docosatetraenoic Acid	0,001	0,018	0,001
C22:5 (n6) cis	cis-4,7,10,13,16-Docosapentaenoic Acid	0,001	0,150	0,001
C22:5 (n3) cis	Docosapentaenoic Acid (DPA)	0,001	0,106	0,001
C22:6 (n3) cis	Docosahexaenoic Acid (DHA)	0,005	2,125	0,010
	Total unknown	0,063	0,220	0,061
	Omega-3 FA	0,433	2,747	0,031
	Omega-6 FA	3,592	2,073	3,988
	Ratio	**8.39: 1**	**1: 1.33**	**235.29: 1**

2.7. General measures

Onset of puberty and bodyweight and length were measured during development at three different developmental time points (4, 6 and 8 weeks old) and at adulthood (10 weeks old). Onset of puberty was determined by assessing the progression of balano-preputial separation (BPS) and scored as either 0 (no separation), 1 (separation but not full) or 2 (full separation). Body length was measured from the tip of the nose to the start of the tail.

2.8. During development

2.8.1. Extended SHIRPA screen (eSH)

This screen has been described elsewhere (Molenhuis et al., 2014). In short, mice were first placed in a circular jar and visually observed. Subsequently, the animal was transferred to a Macrolon Type III cage and video recorded for automated locomotor activity tracking during 5 min (Ethovision 9.0, Noldus Information Technology, Wageningen, The Netherlands). Afterwards, the video was manually scored for grooming behavior using The Observer XT 10.5 (Noldus Information Technology, Wageningen, The Netherlands).

2.8.2. Rota-Rod (RR)

The Rota-Rod (47600, Ugo Basile, Gemonio, Italy) apparatus was used to assess motor coordination and performance. The rotating rod was set to accelerate from 4 to 64 rpm in 5 min and the time on the rod is a measure for (sensori-)motor coordination and balance capacity. The trial was terminated when a mouse fell off or had 2 consecutive turns grasping the rod.

2.9. In adulthood

2.9.1. Open field (OF)

Spontaneous locomotor activity in a novel environment was measured by exposing mice to an open field test. Animals were placed in a circular arena for 15 min. The OF arena had a diameter of 80 cm that, for the analysis, was virtually divided in three equally spaced zones (outer, middle and center zone). Locomotor activity was assessed by video tracking software (Ethovision 7.0, Noldus Information Technology, Wageningen, The Netherlands).

2.9.2. Elevated plus maze (EPM)

Anxiety-related behavior was assessed in the elevated plus maze test based on the natural tendency of rodents to avoid open spaces. Mice were tested on the apparatus, 75 cm above the floor, for 5 min. This was recorded by video tracking software (Ethovision 7.0, Noldus Information Technology, Wageningen, The Netherlands). Time spent on and numbers of entries into each arm, as well as the locomotor activity were measured. Time spent in the arm was measured as the time the animal was inside the arm with all four paws.

2.9.3. Social interest

Mice were allowed to habituate to a clean transparent Macrolon Type II cage with bedding (Tecniplast, Milan, Italy). After 5 min they had a 2-min exposure to a male stimulus animal (A/J inbred strain). This experiment was repeated after 5 min (T5) and 24 h (T24). The time spent exploring the animal was manually recorded using The Observer XT 10 (Noldus Information Technology, Wageningen, The Netherlands).

2.9.4. Home cage screen (HC)

Automated home cage recordings were made to measure novelty-induced and baseline behaviors (Kas et al., 2008). During the experiment of 5 consecutive days, animals are housed individually and total food intake is measured. The experiment was performed as previously described (Molenhuis et al., 2014).

2.9.5. Food burying task

This task assessed the ability of mice to smell volatile odors. Mice were food restricted 24 h before the experiment. After 5 min habituation to the test-environment, Macrolon Type III cages (Tecniplast, Milan, Italy) with double standard bedding material, mice were placed in a clean similar cage with 1 piece of chow hidden underneath the bedding material, in one of the corners, approximate depth was the middle of the bedding. The time to find the buried piece of chow was measured.

2.9.6. Set shifting paradigm

Mice were required to learn the location of a hidden food reward in one of two cups in the test cage (see supplementary Experimental Procedures).

2.10. Statistical analysis

Dietary differences in task parameters were determined using one-way ANOVA (owANOVA). For repeated measurements, a repeated measures ANOVA (rmANOVA) was performed with 'time' as within-subjects factor and 'strain' as between-subjects factor. In case of a significant p-value, post-hoc comparisons were performed using an owANOVA. Not normally distributed data was analyzed using General Linear Measures. Values of $3 \times$ SD above or below the mean were treated as statistical outliers and excluded from further analysis (BTBR; 7 values, BL6; 10 values). SPSS 23.0 for Windows was used for analyses. For lipidomics analyses data processing was performed with XCMS under R version 3.3.2 (Smith et al., 2006; Tautenhahn et al., 2008) and principal component analysis was performed with the R package pcaMethods (Stacklies et al., 2007).

3. Results

3.1. PUFA ratios in the body

Mice exposed to a n-3 deficient or n-3 supplemented diets showed significant changes in their blood and brain n-6/n-3 ratios when compared to mice exposed to the control diet (BTBR; $p = 0.000$, BL6; $p = 0.000$, Fig. 1(A) and (B); detailed statistics Supplementary Table 1). The n-3 deficient diet induced the expected increase in n-6/n-3 PUFA ratio, whereas the n-3 supplemented diet induced the expected decrease in n-6/n-3 PUFA ratio when compared to control diet. The average ratios given in diet (Control (8.4:1), n-3 supplementation (1:1.3) and n-3 deficient (235:1)) were rather similar to the ratios found in whole blood for both BTBR (Control (7.8:1), n-3 supplementation (2.6:1) and n-3 deficient (88.9:1)) and BL6 (Control (6.5:1), n-3 supplementation (2.1:1) and n-3 deficient (88.5:1)).

To establish whether the different diets induced changes in brain lipid composition prior to the onset of behavioral and cognitive studies, brain homogenates from twelve BTBR mice (four mice per diet) were extracted and the lipid extract was subjected to lipidomic analysis by Liquid chromatography – Mass spectrometry(LC/MS) analysis. This resulted in the detection of approximately 300 lipid species. Subsequent principal component analysis of these lipidomes showed a clear distinction to be present between these three groups (Fig. 1(C), left panel). Principal component 1 (PC-1) accounted for 80% of the total variance in these lipidomes and was found to correspond directly to the n-6/n-3 PUFA ratio. The brain lipidomes in the mice fed with the n-3 supplemented diet had notable more similarity to the brain lipidomes obtained with the control diet (groups in relatively close proximity), whereas the n-3 deficient diet resulted in a very dissimilar lipidome as can be concluded from the remoteness of these samples from the control diet lipidomes and, in particular, the n-3 adequate diet (Fig. 1(C)). The second principal component, PC-2, accounted for only 8% of total variance in all samples and did not have any obvious relation to diet.

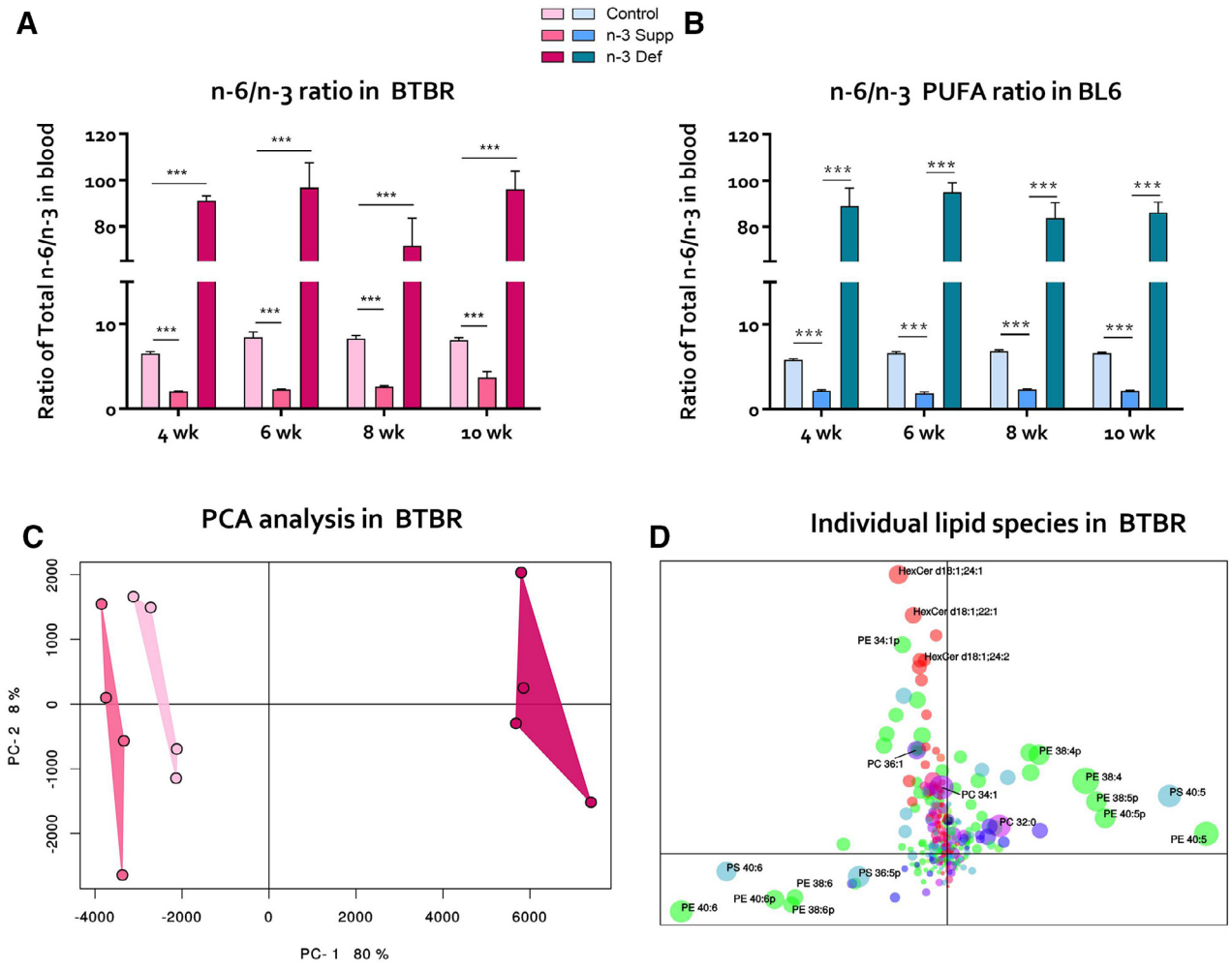

Fig. 1 Dietary n-6/n-3 ratios were reflected in blood samples and brain tissue. (A) and (B) PUFA blood plasma changes in BTBR (A) and BL6 (B) mice exposed to dietary interventions. (C) and (D) Principal Component Analyses (PCA) of brain polar lipid composition in BTBR following dietary interventions. Resulting scores for Principal Components 1 and 2 (PC-1 and PC-2) are depicted in panel C, whereas the loadings of individual lipid species on PC-1 and PC-2 are depicted in the loadings plot in panel D. Lipid species are color coded based on their lipid class. A tailing '*p*' in the lipid name indicates a plasmalogen species. $N = 4$–11 (details Suppl. Table 1). Error bars are depicted as SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

At a more detailed level, the lipids that contributed most to the differences in PC-1 were mainly polyunsaturated Phosphatidylethanolamine (PE) species (visible in the PCA loading plot: Fig. 1(D)). The lipidomes from the n-3 deficient group were enriched in PE 40:5, Phosphatidylserine (PS) 40:5 and PE 38:4 (located at the far-right side). MS/MS (MS2) of these lipid species revealed that they were mainly composed of the n-6 PUFA containing lipid species PE 18:0/22:5, PS 18:0/22:5 and PE 18:0/20:4, respectively. The increase of these n-6 species in the n-3 deficient group was at the expense of the corresponding n-3 species located at the left of the loadings plot: PE 18:0/22:6 (depicted as PE 40:6), PS 18:0/22:6 (PS 40:6) and PE 18:0/20:5 (PE 38:5), respectively. A similar replacement of PUFA was observed in the main ether lipid species: the increased abundance of PE 40:5p (i.e., the plasmalogen PE 18:0/22:5) and PE 38:5p (the plasmalogen PE 16:0/22:5) at the expense of PE 40:6p (i.e., the plasmalogen PE 18:0/22:6) and PE 38:6p (the plasmalogen PE 16:0/22:6).

3.2. Physical development

Longitudinal behavioral assessment across developmental stages (weeks 4, 6, 8, and 10) revealed that dietary intervention influenced body size development in both strains. In BTBR the n-3 supplemented diet reduced bodyweight ($p = 0.000$), whereas in BL6 both n-3 supplemented as well as n-3 deficient interventions reduced bodyweight ($p = 0.000$) (Fig. 2(A) and (B)) the n-3 supplemented diet reduced body length in BTBR ($p = 0.000$) and BL6 ($p = 0.000$) (Fig. 2(C) and (D)). Dietary intervention did not alter glucose levels (BTBR; $p = 0.079$, BL6; $p = 0.119$, Fig. 2(E) and (F)), suggesting that the changes in body weight and body length were not related to metabolic effects. In addition, the n-3 deficient diet increased juvenile brain weight in BTBR ($p = 0.000$, Fig. 2(G)) but had no effect in BL6 ($p = 0.956$, Fig. 2(H)). Both diets delayed puberty onset in BTBR ($p = 0.001$, Supplementary Table 2), whereas the n-3 supplemented diet delayed puberty onset in BL6 ($p = 0.000$,

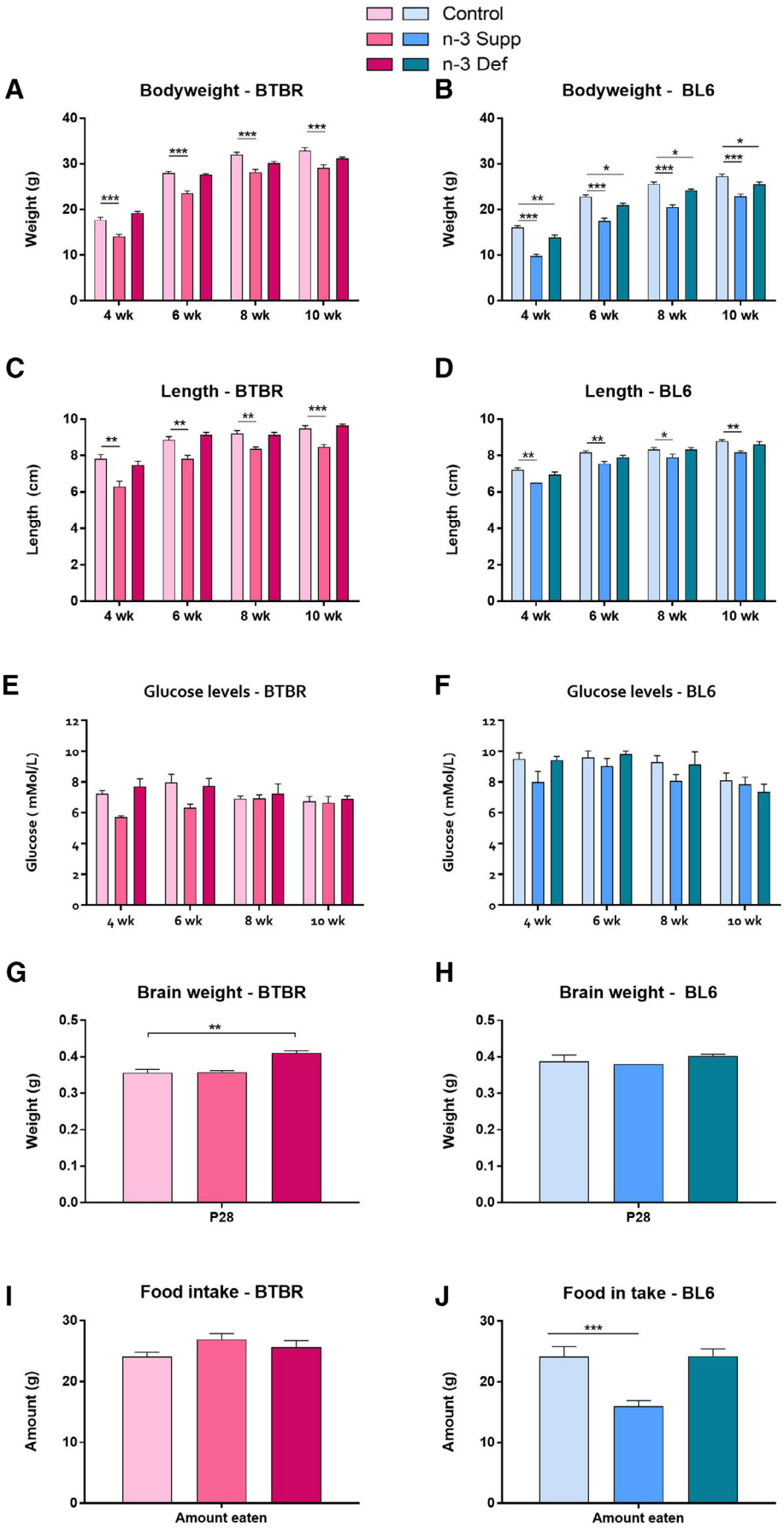

Fig. 2 N-3 PUFA intervention induced developmental changes in body weight and body length. (A) N-3 supplemented BTBR reduced bodyweight. (B) N-3 supplemented and deficient BL6 reduced bodyweight. (C) and (D) N-3 supplemented diet reduced body length in BTBR and BL6. (E) and (F) Glucose levels during development were not affected by diet in BTBR and BL6. (G) Brain weight is higher following chronic n-3 deficiency in BTBR. (H) Brain weight was not affected by diet in BL6. (I) Diet had no effect on food intake in BTBR. (J) The n-3 supplemented diet lowered food intake levels only in BL6. $N = 4$-20 (Details Suppl. Table 1). Error bars

Supplementary Table 3). The n-3 supplemented diet reduced food intake during the 5-day home cage experiment in BL6 mice ($p = 0.000$) but not in BTBR ($p = 0.104$, Fig. 2(I) and (J)). In contrast to measures of puberty onset, body length and body weight, all mice showed normal development of reflexes, muscle strength, and sensory responses following chronic dietary interventions (Supplementary Tables 2 and 3).

3.3. Social behavior

Chronic intake of an n-3 deficiency diet decreased social interest in adult BTBR ($p = 0.002$, Fig. 3(A)) and both n-3 deficient or n-3 supplemented diets decreased social interest in BL6 ($p = 0.012$, Fig. 3(B)). The latter being studied in a second cohort of BL6 mice showing comparable results (Supplementary Figure 1). Both diets did not change the capability to smell a food cue (BTBR; $p = 0.463$, BL6; $p = 0.084$, Fig. 3(C) and (D)), indicating that lack of odor perception is likely not the cause to the reduced levels of social interest following dietary interventions.

3.4. Repetitive and rigid behavior

Dietary intervention had no effect on behavioral and cognitive flexibility. Grooming behavior during development did not change with intervention in both strains (BTBR; $p = 0.411$, BL6; $p = 0.262$, Fig. 3(E) and (F)). Furthermore, reversal learning, assessed in a compound discrimination task during adulthood, were not affected in both lines, indicating that levels of cognitive flexibility were not affected by dietary intervention (BTBR; $p = 0.064$, BL6; $p = 0.219$, Fig. 3(G) and (H)).

3.5. Discrimination capacity and reversal learning

A n-3 supplemented and deficiency diet did not affect cognitive performance in an odor and context specific set-shifting task during adulthood. Both simple and complex discrimination tasks (SD and CD), as well as an extensive intradimensional (IDS I-IVrev) set-shifting task were not affected by diet (BTBR; $p = 0.245$, BL6; $p = 0.219$, Fig. 3(G) and (H)).

3.6. Locomotor behavior

Dietary intervention had no effect on the development of motor balance and sensorimotor functioning in the accelerating Rota-Rod (BTBR; $p = 0.711$, BL6; $p = 0.691$, Fig. 4(A) and (B)). In addition, no dietary effects were observed on motor activity levels during development (BTBR; $p = 0.221$, BL6; $p = 0.027$, Fig. 4(C) and (D)). An n-3 supplemented diet reduced the amount of cage exploration in early life in BL6 ($p = 0.000$) but not in BTBR ($p = 0.304$) (Fig. 4(E) and (F)). In the automated home cage environment, dietary intervention had no effect on light/dark cycle behavioral rhythmicity in BTBR (Light phase; $p = 0.638$, Dark phase; $p = 0.134$, Fig. 4(G)) but in BL6 mice this effect was only in the n-3 supplemented versus n-3 deficient diet comparison (Light phase; $p = 0.008$, Dark phase; $p = 0.023$, Fig. 4(H)). The n-3 supplemented diet reduced novelty-induced motor activity levels during the first hour in the automated home cage environment (BTBR; $p = 0.000$, BL6; $p = 0.000$, Fig. 5(A) and (B)). A n-3 supplemented versus n-3 deficient diet effect was observed in the open field (OF) in BL6, where in both strains there was no dietary effect when compared to the controls (BTBR; $p = 0.585$, BL6; $p = 0.017$, Fig. 5(C) and (D). In the elevated plus maze (EPM), no effects of dietary intervention on motor activity levels were observed (BTBR; $p = 0.985$, BL6; $p = 0.408$, Fig. 5(E) and (F)).

3.7. Anxiety-related behavior

Dietary changes in n-6/n-3 PUFA ratio did not induce anxiety-like behavior in both OF (time spent in center zone: BTBR; $p = 0.656$, BL6; $p = 0.189$, Fig. 5(G) and (H)) and the EPM (time spent in sheltered arms: BTBR; $p = 0.492$, BL6; $p = 0.624$, Fig. 5(I) and (J)).

4. Discussion

This study showed that chronic dietary changes in n-6/n-3 PUFA ratio have a strong impact during mouse development (Table 2). Chronic pre- and postnatal n-3 supplemented or n-3 deficient dietary interventions resulted in a strong developmental delay, reflected by a decrease in bodyweight and body length, and delayed puberty onset in two distinct mouse inbred strains. During adulthood, a wide variety of behavioral and cognitive phenotypes were studied. Despite the strong effects on physical development and puberty onset, dietary interventions did not lead to major changes in adult behavioral and cognitive performance. Interestingly, during adulthood we only observed a reduction in social interest in both strains. Thus, while the fast growing literature is suggesting a potential beneficial role of n-3 PUFAs in the diet (Bernardi et al., 2012; Fedorova and Salem, 2006; Luchtman and Song, 2013; Pietropaolo et al., 2014), the current study shows that chronic pre- and postnatal exposure to altered n-6/n-3 PUFA ratios may have negative impact on development and the expression of adult social behavior in two inbred strains of mice. These findings suggest that dietary n-3 PUFA supplementation should not be considered as beneficial in early developmental stages, in contrast to what has been claimed in literature (Bernardi et al., 2012; Luchtman and Song, 2013). In addition, PUFA interventions should not be considered for the treatment of neurodevelopmental disorders, such as Autism Spectrum Disorders (ASD), unless future studies are able to indicate that these interventions may be beneficial to compensate for potential shifted endogenous PUFA levels in these disorders.

How dietary changes in n-6/n-3 PUFA ratio lead to developmental delay remains to be investigated. Interestingly, n-3 deficient and n-3 supplementation led to different brain fatty acid compositions (Fig. 1(D)), and both led to developmental delay (Table 2), indicating that brain lipid composition changes (irrespective of their direction) may be disruptive for normal developmental processes. Unfortunately, there is a large heterogeneity in literature on the effects of dietary n-6/n-3 PUFA intervention on development of the

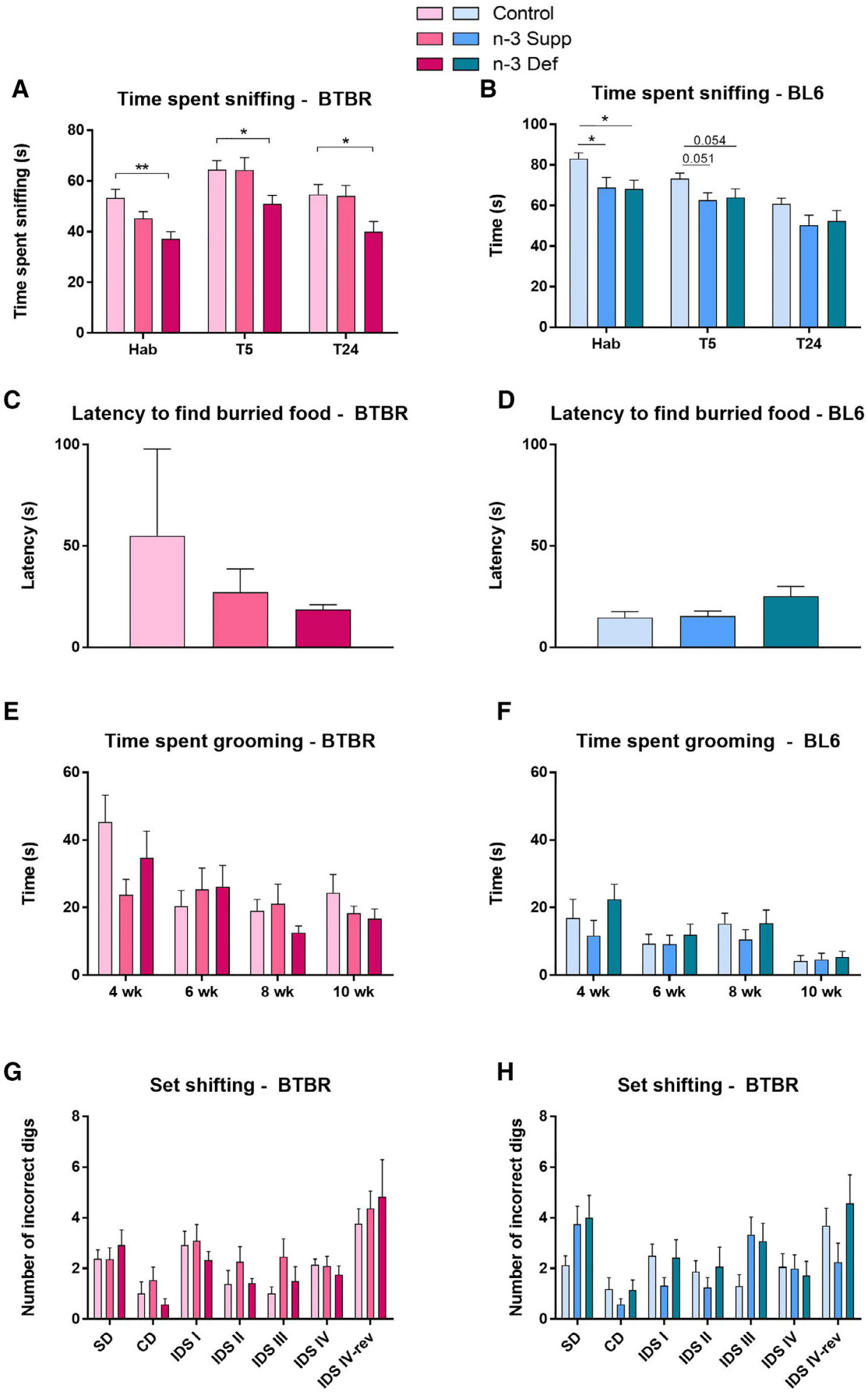

Fig. 3 Social, repetitive and cognitive behavior during adulthood. (A) N-3 deficiency reduced social interest in BTBR at 3 timepoints. (B) n-3 supplemented and n-3 deficient fed BL6 reduced social interest at 2 timepoints. (C) and (D). The ability to smell was not affected in BTBR and BL6. E. No differences in time spent grooming with each diet in BTBR and BL6. (G) and (H). Cognitive flexibility was not affected in BTBR and BL6 following dietary interventions (abbreviations: simple discrimination (SD), complex discrimination (CD), Intradimensional shift (IDS), 4th Intradimensional reversed shift (IDS IV-rev). $n = 5$–20 (Details Suppl. Table 1).

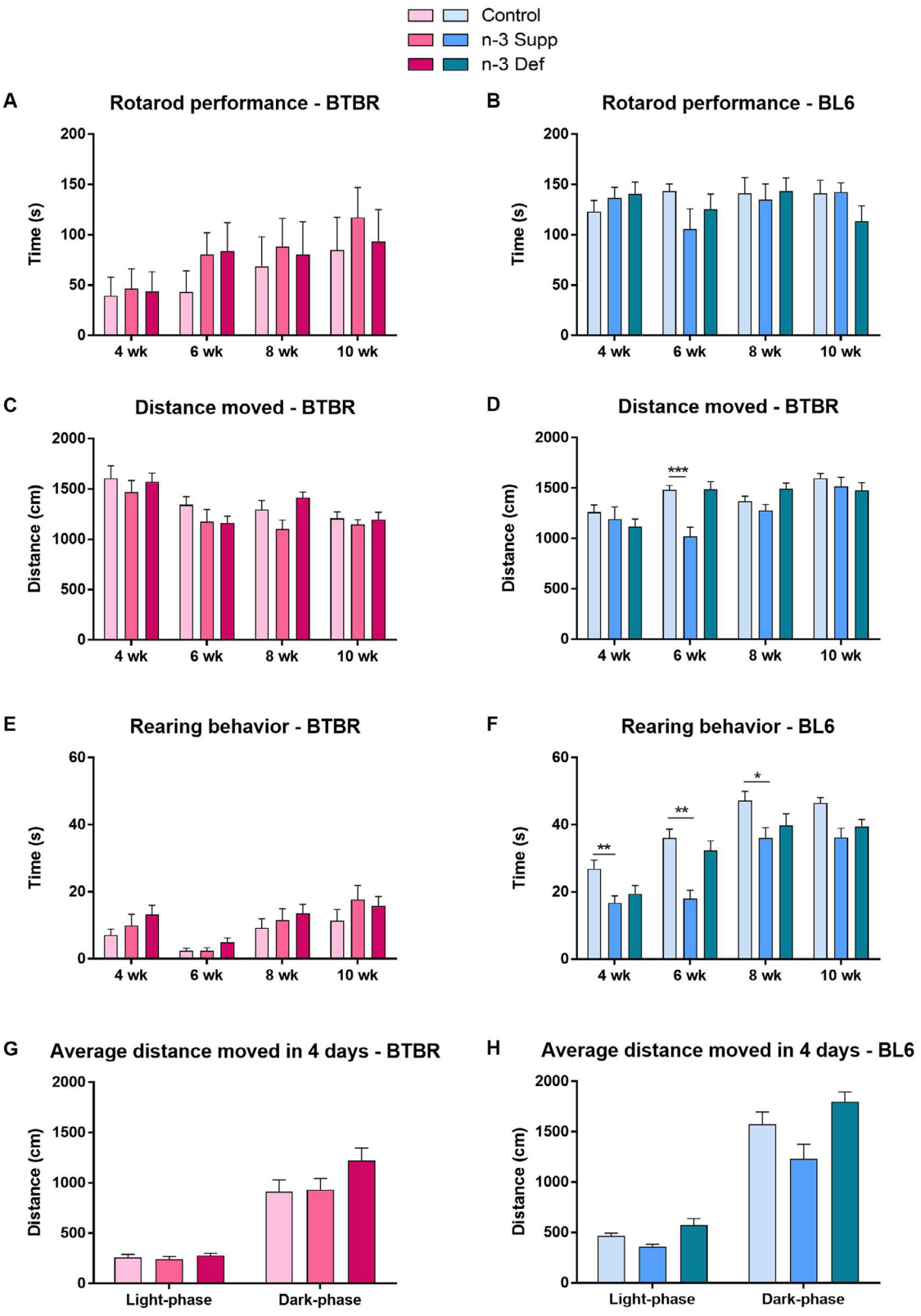

Fig. 4 Development of locomotor behavior. (A) Rota-Rod performance was not affected in BTBR and BL6. (C) and (D). No difference in distance moved during development in BTBR and BL6. (E) No difference in rearing behavior during development in BTBR. (F) N-3 supplementation fed BL6 reduced rearing behavior during early development. (G) (H). No difference in distance moved during 4 days in BTBR and BL6 compared to the control group. $N = 9$–16 (Details Suppl. Table 1). Error bars are depicted as SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

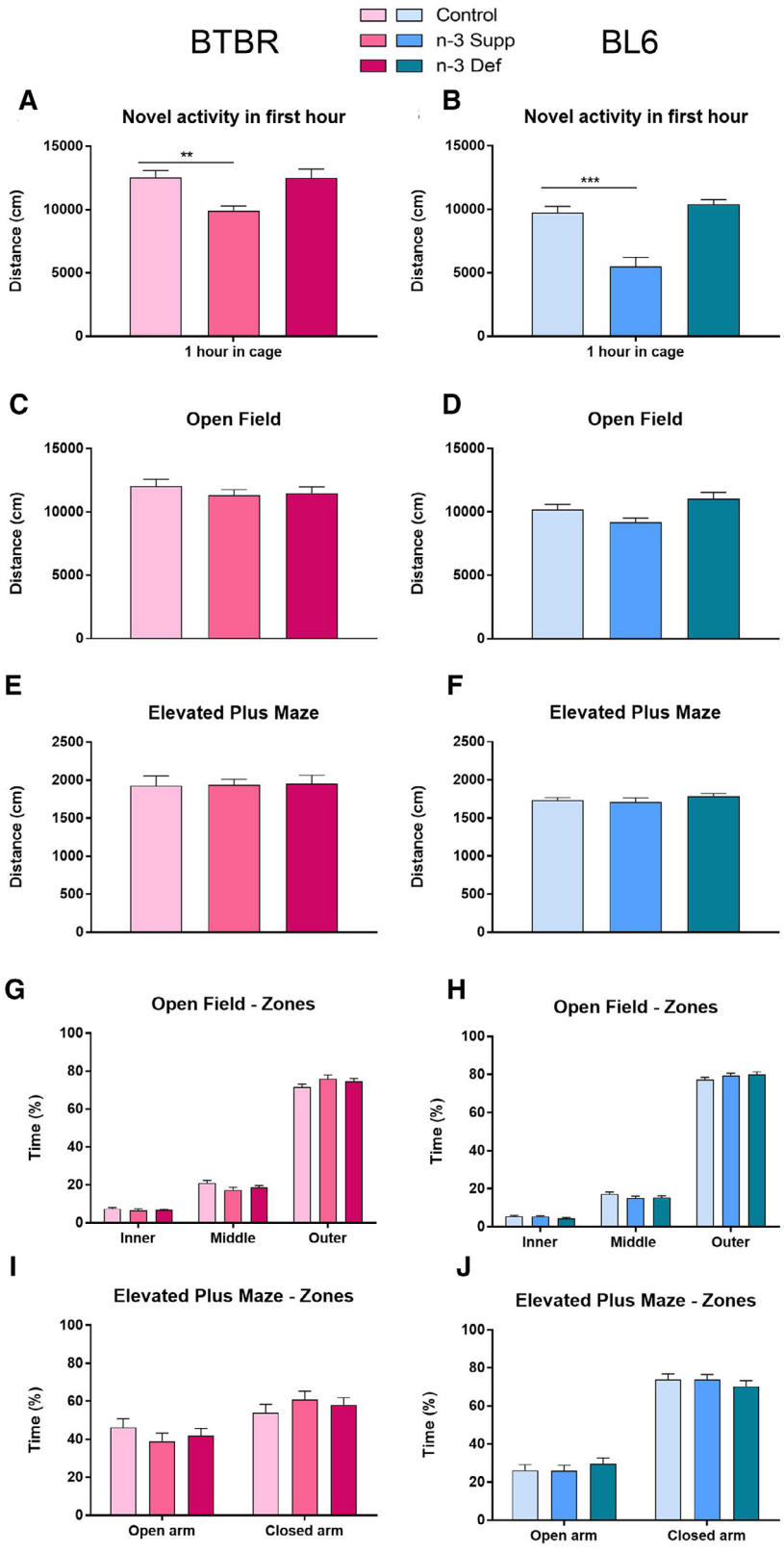

Fig. 5 Locomotor behavior and anxiety during adulthood. (A) and (B). The n-3 supplemented diet reduced novel activity in the home cage in BTBR and BL6. (C) and (D). No difference in open field activity levels in BTBR and BL6. (E) and (F) No difference in motor activity levels of BTBR and BL6 in the EPM (G) and (H). No effect of diet on time spent in zones in open field in BTBR and BL6. (I) and (J) No difference in time spent in arms in EPM in BTBR and BL6. $N = 10$–16 (Details Suppl. Table 1). Error bars are depicted as SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 2 Heatmap of the effects of chronic dietary n-6/n-3 PUFA ratio changes observed in the present study. The heatmap visualizes all measured effects of PUFAs on the (developmental) outcome of BTBR and C57BL/6J mice. The darker the color, the more significant the effect of PUFAs on this measure. Red = negative effect (i.e., quantitative reduction; qualitative negative effect in case of brain weight and puberty onset), Beige = no effect.

Domain	Measure	n-3 Supplemented		n-3 Deficient	
		BTBR	BL6	BTBR	BL6
Development	Bodyweight				
	Length				
	Onset of Puberty				
	Brain weight juvenile				
Metabolic	Blood glucose				
	Total food intake				
	Weight loss after 24h				
Social	Social interest				
	Capability to smell				
Repetitive	Grooming behavior				
	Reversal learning				
Cognition	Simple discrimination				
	Complex discrimination				
	Intradimensional shift				
Locomotor	RotaRod				
	Distance moved Open Field				
	Activity in development				
	Distance moved EPM				
	Circadian rhythm Home Cage				
	Novel activity in Home Cage				
	Rearing development				
Anxiety	EPM				
	OF				

body and reflexes (Amusquivar et al., 2000; Bongiovanni et al., 2007; Carrié et al., 2000; Fountain et al., 2008; Hilakivi-Clarke et al., 1997; Korotkova et al., 2005, 2002; Lamptey and Walker, 1976; Pietropaolo et al., 2014; Santillán et al., 2010; Troina et al., 2010; Wainwright et al., 1997; Weiser et al., 2016; Xiang and Zetterström, 1999) which could, in part, be due to differences in study design. Interestingly, and in line with our findings, several studies found that PUFA dietary interventions either led to body weight changes, reduced body length, or delayed puberty onset, indicating that PUFA interventions may affect developmental delay (see supplementary Table 4). However, none of these studies assessed all these three measurements at different development stages. Therefore, our study is the first showing that *chronic* PUFA interventions with n-3 supplementation leads to developmental delay on the basis of all three measures (body weight, body length and puberty onset) that were all measured at four different time points during development. In addition, we did confirm that the developmental delay was not a consequence of affected locomotor or repetitive behavior in studies with similar and different intervention durations and ratios (Fortunato et al., 2016; Fountain et al., 2008; Pietropaolo et al., 2014; Wu et al., 2016) and literature suggests that changed behavior seems to be more affected by PUFA ratio than individual levels (Korotkova et al., 2005). Several studies indicated pathways through which this developmental delay may be established. First, PUFA ratio changes may lead to metabolic changes and thereby altering body gain (Korotkova et al., 2005). We, and others, found no changes in glucose levels following PUFA interventions (Bjursell et al., 2014; Korotkova et al., 2005, 2002), indicating that a change in n-6/n-3 ratio has no direct effect on glucose levels. However, a reduction in fasting insulin levels was previously reported, without affecting blood glucose levels (Bjursell et al., 2014; Korotkova et al., 2005, 2002). Next to metabolic changes, changing the n-6/n-3 ratio may also influence signal transduction as PUFAs are ligands for peroxisome proliferator-activated receptors (PPARs) (Abbott, 2009). Expression of different PPARs are related to the n-6/n-3 ratio in diet (Hajjar et al., 2012; Tian et al., 2011). However, for the current study the influence of PUFAs on PPARs remains to be investigated. Third, PUFA ratio changes may result in inhibition of growth, as the present study found both shorter and lighter animals, as well as a delayed puberty onset. The reduced food intake in the C57BL6J group may be the result of taste preferences, but our other experimental groups on similar dietary intervention did not show this reduced food intake. Previous studies suggest that n-3 supplementation feedings reduced length (Santillán et al., 2010),

body fat mass (Troina et al., 2010) and subsequent related changes in puberty onset (Santillán et al., 2010; Troina et al., 2010), but were not able to obtain similar findings on body weight development, despite the rather similar experimental design and interventions. Lastly, the developmental delay could be due to a change in cell proliferation; literature suggests that neurogenesis is altered in the embryonic rat brain when exposed to a high n-6/n-3 PUFA ratio (Coti Bertrand et al., 2006; Kawakita et al., 2006). On the contrary, the brain weight differences in the current study were only reported in the n-3 depleted BTBR, without any developmental delay. Brain volume is reduced and cell migration is transiently delayed when given an n-3 deficient diet, but the sensitive period for these PUFA effects is unknown yet (Bernardi et al., 2012; Coti Bertrand et al., 2006; Yavin et al., 2010). It is well known that the last trimester of pregnancy and first 6 months of human life are most important for n-3 PUFA uptake (Bernardi et al., 2012; Guesnet and Alessandri, 2011; Hamosh and Salem Jr., 1998; van Elst et al., 2014). However, up to now, there is no consensus on which pre- or postnatal time point influences the developmental delay the most (Amusquivar et al., 2000; Moriguchi and Salem, 2003), especially since not many studies investigated all three points of developmental delay; bodyweight, length and puberty onset; in one experiment (Supplementary Table 4) Given these points, we propose that especially the timing of PUFA intervention and the effects on fat mass and cell proliferation should be considered in future research to investigate the impact of PUFAs on developmental processes.

It is remarkable that dietary changes in n-6/n-3 PUFA ratio have very limited impact on adult behavioral and cognitive performance, while the treated mice in the present study all suffered from a significant developmental delay before reaching adulthood. These findings suggest that early developmental impairments can be compensated for when reaching adulthoods. Alternatively, dietary changes in n-6/n-3 PUFA ratio may lead to developmental delay during the early stages in life, but may have beneficiary effects on outcome during adulthood; providing a possible explanation why animals on a n-3 supplemented of n-3 deficient diet catch up later in life. Furthermore, it is remarkable that animals with a developmental delay showed normal levels of cognitive functioning in our study, although this was not confirmed in literature (Carrié et al., 2000; Catalan et al., 2002; Fountain et al., 2008; Greiner et al., 1999; Lamptey and Walker, 1976; Moriguchi and Salem, 2003; Robertson et al., 2017; Weiser et al., 2016; Wu et al., 2016; Yamamoto et al., 1988; Yonekubo et al., 1993), and for this reason it should be noted that the experimental design of all these studies were different to each other (supplementary Table 4). Next to that, we found normal adult behavioral performance, except for their levels of repeatedly measured adult social behavior. These findings may suggest that chronically altering n-6/n-3 PUFA ratios may affect brain circuitry involved in social behavioral regulation. Furthermore, it may also suggest that the developmental delay leads to disrupted social behavior during the juvenile stage that is known to lead to abnormal adult social behavioral expression (Hol et al., 1999). On the contrary, abnormal developmental delay led to normal adult social behavior in n-3 depleted BL6, whilst no developmental delay led to abnormal social behavior in n-3 depleted BTBR. The latter group did have a changed brain volume, which may result in abnormal social behavior. These limited effects of dietary treatment in adulthood indicate that additional experiments are needed to further investigate the underlying causes on the relation between developmental delay and the limited behavioral deficits in adulthood.

Thus far, no other animal studies using PUFA interventions have reported on the strong delay in development that we observed in the present study. This could be a consequence of the chronic nature of our intervention strategy (both pre- and post-natal) in combination with relative strong differences in PUFA ratios when compared to about half of the earlier published studies (see supplementary Table 4). In studies with similar high levels of PUFA interventions, no signs of developmental delay have been reported in a similar fashion as in the present study (see supplementary Table 4). Most of these studies, however, have not studied phenotypes in a longitudinal manner, and have not studied the onset of puberty, making it very unlikely to find comparable results to the present study. The differences in body weight in the present study could not be due to differences in the caloric content of the three diets, as the caloric contents are very similar (Control diet: 3.895 Kcal/g, n-3 depletion diet: 3.893 Kcal/g; n-3 supplementation 3.896 Kcal/g). Similar to our observations, studies in humans also observed that higher intake of omega-3 resulted in a significant bodyweight loss, indicating that PUFA intake alters body composition in humans as well (Bender et al., 2014). Additional animal studies with chronic intake of lower PUFA ratios, as well as studies during which only pre-natal versus only postnatal PUFA interventions are given will be needed to better understand the impact of chronic treatment on relatively high PUFA ratios on developmental delay.

Together, the current study shows that two very different mouse inbred strains that are similarly exposed to different levels of n-6/n-3 PUFA levels both express a developmental delay and reduced adult social interaction with little behavioral and cognitive effects in later life. This is remarkable, as the mechanistical data shows that there indeed are very profound differences between the intervention groups on the individual fatty acid level in the brain. Even more, these effects were found regardless of genetic background; the comparison between BTBR and BL6 has not been described previously. Future studies should be designed in a similar manner to increase our knowledge on the particular effects of dietary n-6/n-3 ratio changes, to independently replicate the effects from this study, and to be able to develop follow-up studies investigating the most optimal n-6/n-3 PUFA ratios during pre- and postnatal periods. Indeed, there seems to be relevance in investigating these dietary effects more in relation to physical development, such as body composition and puberty onset, and development of adult social behavior. Overall, the present findings indicate that chronic dietary supplementation or depletion of n-3 PUFA's may not be beneficial.

Conflict of interest

All authors declare no conflicts of interest. BB was fully employed by Vifor Pharma.

Contributors

KvE Designed and performed experiments, took care of animals, analyzed and interpreted data, and wrote the manuscript, JFB Performed experiments, analyzed data and edited the manuscript, JEM Performed experiments, analyzed data and took care of animals, MHB Performed experiments and took care of animals, BB Edited the manuscript, JBH Designed experiments and edited the manuscript, MJHK Designed experiments, interpreted data and edited the manuscript. All authors contributed to and have approved the final manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2018.11.1106.

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